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Molecular Determinants of cGMP Binding to Chicken Cone Photoreceptor Phosphodiesterase*

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Daming Huang‡, Thomas R. Hinds‡, Sergio E. Martinez‡, Catalin Doneanu§, and Joseph A. Beavo‡¶

From the Departments of ‡Pharmacology and §Medicinal Chemistry, University of Washington, Seattle, Washington 98195

Structural studies on photoreceptor phosphodiesterases type 6 (PDE6s) have been hampered by an inability to express and purify substantial amounts of enzyme. Here we describe bacterial expression and characterization of the chicken cone PDE6 regulatory GAF-A and GAF-B domains. High affinity cGMP binding was found only for GAF-A as predicted from sequence alignments with the GAF domains of PDE2 and PDE5. A homology model of the GAF-A domain of chicken cone PDE6 based on the crystal structure of mouse PDE2A GAF-B was used to identify residues likely to make contact with cGMP. Alanine mutagenesis of 4 of these residues (F123A, D169A, T172A, and T176A) showed that each was absolutely required for cGMP binding. Three of these residues map to the H4 helical structure of the GAF-A domain indicating this region as a key structural component for cGMP binding. Mutagenesis of another residue, S97A, decreased cGMP binding affinity 5-fold. Finally mutagenesis of Glu-124 indicated that it is responsible for part but not all of the high specificity for cGMP binding to PDE6 GAF-A. Since little data is available on the properties of the chicken cone PDE6 holoenzyme, we also characterized the native PDEs of chicken retina. Two histone-activated PDE6 peaks were separated by ion exchange chromatography and identified by mass spectrometry as cone and rod photoreceptor PDE6s, respectively. Both of these PDEs had cGMP binding and kinetic properties similar to their corresponding bovine photoreceptor PDEs. Moreover the cGMP binding properties of chicken cone PDE6 holoenzyme were very similar to those of the bacterially expressed individual GAF-A or GAF-A/B domains.

There are two classes of photoreceptors, cones and rods, that differ substantially in their response to light (1–3). Rods are very sensitive to low levels of light and can respond to a single photon. Cones are about 100 times less sensitive but respond faster, and the light signal can be terminated more rapidly than in rods (3, 4). Both rod and cone photoreceptors can sense and respond to changes of light through a G-protein-mediated signaling cascade that activates a family of cGMP-specific phosphodiesterases, PDE6s.[1] Activation of these PDEs decreases the level of cGMP and thereby modulates the activity of a cyclic nucleotide-gated cation channel. This in turn controls the release of neurotransmitter from the photoreceptor neuron. Therefore, PDE6s play a crucial role in both rod and cone phototransduction.

The rod PDE6 holoenzyme has been characterized as a heterotetramer containing one α-subunit (PDE6A), one β-subunit (PDE6B), and two γ-subunits (5). Cone PDE6 is composed of two identical α'-subunits (PDE6C) and two γ-subunits (6). The α-, β-, and α'-subunits contain the catalytic sites that hydrolyze cGMP. The γ-subunits bind to the holoenzyme and inhibit phosphodiesterase activity. Cone and some rod PDE6s also contain a δ-subunit that may target the normally membrane-bound PDE6 to the cytosol (7, 8). However, the δ-subunit is also a component of several other proteins and therefore not unique to photoreceptors (8).

Although in most vertebrate retinas, cones are much less abundant than rods, the chicken retina is cone-dominant. In this species, cones outnumber rods six to one in the central retina and three to one in the peripheral retina (9). Most previous biochemical studies of the cone PDE6 isoenzyme have been carried out using bovine retinas due to their large size and availability. Nevertheless a cDNA predicted from homology arguments to encode the chicken cone PDE6 α'-subunit has been isolated and characterized (10). Similarly a cDNA most homologous to the bovine PDE6 β-subunit cDNA also has been reported from chicken pineal gland (11).

PDE6 is a member of the 11 families of Class 1 phosphodiesterases that hydrolyze cyclic nucleotides. Five of these PDE families, PDEs 2, 5, 6, 10, and 11, contain one or two complete GAF domains in their N-terminal regulatory regions (12, 13). GAF domains are regulatory small molecule-binding domains originally named for their presence in cGMP-regulated PDEs, certain adenylyl cyclases and the transcription factor FhlA of bacteria (14). Cyclic GMP binds to one of two GAF domains of PDE2, PDE5, and PDE6 (6, 15, 16). The catalytic activity of PDE2A is allosterically stimulated by cGMP binding to its GAF-B domain (15). In PDE5, cGMP binding to the GAF-A domains increases PDE5 catalytic activity and potentiates phosphorylation at an N-terminal serine (17, 18). The functions, regulation, and roles of GAF domains in PDEs have been comprehensively reviewed recently (19).

For the amphibian photoreceptor PDE6, it has been found that cGMP occupancy at a GAF domain enhances P-γ binding to the holoenzyme (20, 21). Several roles for cGMP binding have been proposed. For example, it has been postulated that non-catalytic cGMP binding to PDE6 may be involved in the recovery from light stimulation and light adaptation (22). In

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¶ To whom correspondence should be addressed: Dept. of Pharmacology, University of Washington, Seattle, WA 98195. Tel.: 206-543-4006; Fax: 206-685-3822; E-mail: beavo@u.washington.edu.

[1] The abbreviations used are: PDE, 3′,5′-cyclic-nucleotide phosphodiesterase; GAF, cGMP-regulated PDEs, *Anabaena* adenylyl cyclase, *E. coli* protein FhlA; IBMX, 3-isobutyl-1-methylxanthine; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high pressure liquid chromatography; H4, helix α-4.

this case, the GAF sites serve as a cGMP reservoir to buffer cytoplasmic cGMP levels in the dark and accelerate the return of high cGMP to basal levels upon light activation of the PDE. Another model suggests that cGMP binding to a GAF domain regulates the duration of transducin activation of PDE6 by modulating the affinity of P-γ (23, 24). These models are not mutually exclusive.

Recently the three-dimensional structure of the mouse PDE2A GAF-A/B domains was determined by x-ray diffraction crystallography at 2.9-Å resolution (25). The regulatory PDE2 GAF-A/B domains form a parallel dimer in which only GAF-B binds cGMP. There are 11 amino acid residues that make contact with cGMP and line the binding pocket. In PDE5, 10 of these 11 residues are identical in GAF-A, which therefore allowed the prediction that this would be the cGMP-binding domain of this PDE (25). It was subsequently confirmed that GAF-A of PDE5 is sufficient for high affinity cGMP binding (26). A consensus cGMP-binding motif, based on the similarity between PDE2A GAF-B and PDE5 GAF-A domains, has been proposed (Sequence 1, Ref. 25). Mutagenesis in this motif in

S X(13-18)	**FD** X(18-22)	**IA** X(21)	**[Y/N]** X(2)	**VD** X(2)	**T** X(3)	**T** X(19)	**[E/Q]**
1	2,3	4,5	6	7,8	9	10	11

SEQUENCE 1

PDE5 suggested that the Phe in the FD dyad of PDE5 GAF-A domain is essential for cGMP binding (27). A similar finding has also been shown for the PDE2A GAF-B domain (28). Eight of the 11 residues in mouse PDE2A, (*i.e.* Ser-424, Phe-438, Asp-439, Val-484, Asp-485, Thr-488, Thr-492, and Glu-512) contact cGMP via side chains. The crystal structure of PDE2 GAF-B and recent mutagenesis studies suggest that its ability to discriminate cGMP *versus* cAMP is largely determined by Asp-439, which provides positive specificity for cGMP binding via hydrogen bonds between its main chain NH, side chain carboxyl, and the O-6 and N-1 positions of the guanine base of cGMP (25). This residue also provides a negative determinant for cAMP (28).

Compared with PDE2 and PDE5, most PDE6s have both higher binding affinity and higher specificity for cGMP (6, 29). Bovine rod PDE6 binds cGMP with K_d values reported from 25 to 500 nM at a low affinity binding site (23) and <500 pM at a high affinity site (30). Bovine cone PDE6C appears to have one type of cGMP-binding site with a K_d of about 10 nM (7). A chimeric bovine PDE6C/PDE5 enzyme also has been reported to contain a single class of non-catalytic cGMP-binding sites with a K_d of 450 nM (31).

In this study we report on the bacterial expression of the tandem GAF domains of chicken cone PDE6 and show that the basic features of the cGMP-binding pocket is highly conserved between the cone PDE6C and mouse PDE2A GAF domains. We demonstrate that GAF-A instead of GAF-B contains the single high affinity cGMP-binding domain of PDE6C and that the conserved residues of PDE6C (Phe-123, Asp-169, Thr-172, and Thr-176) each appear to play significant roles in forming a functional cGMP-binding pocket. Finally we report on the isolation and characterization of the chicken PDE6 holoenzymes and show that their cGMP binding characteristics are similar to those of the isolated chicken GAF domains and also to their corresponding mammalian PDE6 counterparts.

EXPERIMENTAL PROCEDURES

Materials

Chicken eyes were obtained from the Tyson Co. (Little Rock, AK). Bovine eyes were purchased from Schenk Packing (Stanwood, WA). [^{3}H]cGMP was obtained from Amersham Biosciences; cGMP, 3-isobutyl-1-methylxanthine (IBMX), isopropyl β-thioglucopyranoside were from Sigma. *Pfu* DNA polymerase and the QuikChange® site-directed mutagenesis kits were obtained from Stratagene (La Jolla, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Methods

Isolation and Initial Purification of Chicken Rod and Cone PDE6 Holoenzyme—In a typical experiment 50–100 chicken retinas were dissected in the light, separated from much of the vitreous humor, and homogenized in hypotonic buffer (10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) using a dozen strokes of a motor-driven Teflon pestle in a glass homogenizer (Potter-Elvehjem tissue grinder). The homogenate was centrifuged at 100,000 × *g* for 1 h. The supernatant was applied to a 75-ml DE52 anion-exchange column and eluted with a linear NaCl gradient (20–300 mM NaCl, 10 mM Tris, pH 7.5, 1 mM $MgCl_2$) run at a flow rate of 1 ml/min at 4 °C. Sixty fractions of 5 ml each were collected at 4 °C and assayed for histone-activated (2.5 mg/ml, type VIII-S) phosphodiesterase activity by measuring the release of phosphate (7).

cGMP Affinity Column Purification of Chicken PDE6 Holoenzyme—Two histone-activated PDE activity peaks of approximately equal activity were separated on the DE52 anion-exchange column. Approximately 35 ml of the first histone-activated PDE6 peak was pooled and loaded onto an epoxy-Sepharose cGMP affinity column (7, 15). The column was washed two times with low salt buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 10 mM dithiothreitol, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and the cGMP-binding proteins, including chicken photoreceptor PDE, were eluted with 1 mM cGMP in the presence of 2 mM EDTA and 1 mM IBMX in low salt buffer at room temperature. The presence and purity of chicken photoreceptor PDEs were analyzed by SDS-PAGE and silver staining.

Immunoprecipitation of Chicken PDE6s—It has been shown previously that the ROS-3 monoclonal antibody recognizes both rod and cone photoreceptor PDE from bovine retina (32). The putative chicken cone and rod PDE6 holoenzymes, corresponding to the first or the second histone-activated PDE6 peaks, were immunoprecipitated using the ROS-3 monoclonal antibody bound to Protein G. Typically 15 ml of the DE52 fraction of either chicken rod or cone PDE was mixed with 200 μl of antibody resin (500 μg of antibody bound to 200 μl of Protein G PLUS-agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C) in the presence of 150 mM NaCl. After mixing overnight, the resin was pelleted and washed three times with 1 ml of 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 300 mM NaCl at 4 °C.

Identification of Chicken Rod and Cone PDE6 Using Mass Spectrometry—On-line nano-liquid chromatography/electrospray ionization-MS/MS experiments were performed on an API-US quadrupole time-of-flight mass spectrometer (Micromass) equipped with the CapLC system (Waters, Milford, MA). The stream select module was configured with an OPTI-PAK Symmetry300 C_{18} trap column (Waters) connected in series with a nanoscale analytical column (75-μm inner diameter × 15 cm, packed with 3.5 μm, XTerra MS C_{18} particles (Waters)).

The eluate from the cGMP affinity column was concentrated to 100 μg/ml using a Centriprep centrifugal filter (Millipore) and then digested with 5 μg/ml trypsin. In other experiments immunoprecipitated proteins were separated by SDS-PAGE and stained with Coomassie Blue. The protein bands corresponding to the molecular weights of rod and cone PDE6 were sliced out from the gel and digested with trypsin for analysis by mass spectrometry (33). Protein digests (5 μl) were injected onto the trap column at 10 μl/min, desalted, and back-flushed to the analytical column at 0.5 μl/min using a gradient elution. The gradient consisted of 5–50% solvent B for 30 min followed by 50% B for 15 min and 50–90% B for 5 min (A = 5% acetonitrile, 0.1% formic acid; B = 95% acetonitrile, 0.1% formic acid).

Quadrupole time-of-flight parameters were set as follows: the electrospray potential was set to 3.5 kV, the cone voltage was set to 60 V, the extraction cone was set to 2 V, and the source temperature was set to 80 °C. The MS survey scan was *m/z* 400–1600 with a scan time of 1 s, and the collision energy was set to 10 eV. When the intensity of a peptide peak rose above a threshold of 20 counts, tandem mass spectra were acquired using the data-dependent algorithm implemented in the MassLynx acquisition software. For operation in the MS/MS mode, the scan time was increased to 2 s, the isolation width was set to include the full isotopic distribution of each peak (3 Da), and the collision energy was set to 15–25 eV. MS/MS spectra were recorded for the doubly, triply, and quadruply charged molecular ions of peptides. All MS/MS spectra were searched against the non-redundant National Center for Biotechnology Information protein data base by using MASCOT (34) assuming a mass tolerance of 0.3 Da for both the precursor and the fragment ions.

cGMP Binding to the Chicken Rod and Cone PDE6 Holoenzymes—Chicken cone PDE6 holoenzyme was immunoprecipitated as described previously except less sample and antibody were used. Usually 1–2 ml of DE52 fraction of chicken rod or cone PDE was mixed with 100–200 μl of antibody resin (100–200 μg of antibody). After incubation overnight, the resin was collected by centrifugation and washed three times with 1 ml of 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 300 mM NaCl. The PDE immunoprecipitates were then incubated with 5 mM Tris, pH 7.5, 25 mM NaCl, 2 mM EDTA, 1 mM IBMX, 0.1 mg/ml bovine serum albumin, 2–600 nM (7.5 Ci/mmol) [^{3}H]cGMP (200-μl total volume) for 2 h at room temperature. [^{3}H]cGMP bound to the immunoprecipitated PDE was separated from free ligand by filtration on Millipore filters (0.45-μm HA). The Millipore filters were dissolved in Filter-Count® complete liquid scintillation counting mixture (PerkinElmer Life Sciences) overnight and counted in a Packard 1600 TR liquid scintillation analyzer.

cGMP Binding and cAMP Binding by Individual Chicken Cone PDE6 GAF Domain Proteins—The binding of cGMP or cAMP to GAF domain proteins was analyzed by the Millipore filter binding assay as described previously (37) usually as a competition assay (28). For the cGMP binding competition assays, 1 nM GAF domain protein was incubated with a fixed amount of [^{3}H]cGMP (usually 1 nM) and various amounts of unlabeled cGMP from 2 to 600 nM for 2 h at room temperature. For the cAMP binding competition assays, 1 nM GAF domain protein was incubated with 1 nM [^{3}H]cGMP and various amounts of unlabeled cAMP from 1 μM to 100 mM for 2 h at room temperature.

Most K_d values reported in this study were determined by the homologous or heterologous displacement methods. Care was taken to utilize concentrations of protein that were lower than the measured K_d so that the data represented true binding curves and not a titration analysis. Similarly for all IC_{50} determinations the concentrations of labeled radioligand was adjusted to be lower than the measured IC_{50} value so that the IC_{50} approached the K_i for the cold ligand (35, 36). Therefore, the affinity of the ligand can be calculated using the equation of Cheng and Prusoff (35) that states that the equilibrium dissociation constant of the ligand, $K_i = IC_{50}/(1 + [\text{Radioligand}]/K_d)$. In the case of homologous displacement, *e.g.* [^{3}H]cGMP being displaced by cGMP, the equation further simplifies to $K_i = IC_{50} - [\text{Radioligand}]$ since $K_i = K_d$. Curve fitting was done using GraphPad Prism4 with a one-site competition model constraining the 100 and 0% binding points. Better fits were not obtained with a multiple site model.

Enzyme Kinetics of Chicken Rod and Cone PDE6 Holoenzymes—The kinetics for both cGMP and cAMP hydrolysis of the chicken rod and cone PDE6 were determined using fractions from the ion-exchange column. PDE6 activity was assayed in the presence of histone (2.5 mg/ml, type VIII-S, Sigma) by PDE activity assay using either [^{3}H]cGMP or [^{3}H]cAMP as substrate (15, 32). The K_m values for cGMP and cAMP hydrolysis were derived by nonlinear regression analysis using GraphPad Prism® from data points obtained using 1–250 μM [^{3}H]cGMP or 50–2500 μM [^{3}H]cAMP as substrate.

Three-dimensional Structure Model of Chicken Cone PDE6 GAF-A Domain—A three-dimensional model for the chicken PDE6 GAF-A domain was constructed based upon the crystal structure of the mouse PDE2A GAF-B domain using the Swiss-Model program (38). Sequence alignments were made with the ClustalW program (39). After adding side chains from a rotamer data base, the working model was energy-minimized using GROMOS 96 (38).

Cloning, Expression, and Purification of the GAF Domains of Chicken Cone PDE6—The p^{RunH} plasmid (a derivative of the pMW172 vector (40)) containing a C-terminal His_6 tag was used as an expression vector for the GAF-A, GAF-B, and GAF-A/B domains of chicken PDE6C. Full-length chicken PDE6C cDNA was a generous gift from Dr. Semple-Rowland (University of Florida). The boundaries of GAF-A/B, GAF-A, and GAF-B domain constructs were amino acids 42–458, 42–238, and 248–458, respectively, based on homology alignment with other PDE GAF domains. DNA coding for these domains was PCR-amplified using the full-length chicken PDE6C as template and primers containing BamHI and XhoI sites. The PCR products were ligated into the p^{RunH} vector downstream of the T7 promoter.

Chicken cone PDE6 GAF domain constructs were transformed into C41 competent *Escherichia coli*. Protein expression was induced by isopropyl β-thioglucopyranoside at 16 °C, and cells were grown overnight. The cell pellets were resuspended in lysis buffer (100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 10 μg/ml DNase I, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and disrupted through either a French Press or a Microfluidizer® cell disruption apparatus. The lysates were centrifuged at 10,000 × *g* for 1 h at 4 °C, and the supernatant was incubated with Talon® resin (Clontech) for 2 h at 4 °C. The bound His_6-tagged proteins were eluted with buffer containing 150 mM imidazole, 50 mM sodium phosphate (pH 7.0), and 300 mM NaCl. The eluted proteins were concentrated using a Centriprep centrifugal filter (Millipore) and subjected to gel filtration using a Superose-12 column (Amersham Biosciences) to remove aggregated protein and determine the apparent molecular weight of the GAF proteins. SDS gel electrophoresis was carried out to determine the purity of the isolated GAF domain proteins.

Site-directed Mutagenesis Studies on Chicken Cone PDE6C GAF-A and GAF-A/B Domains—The QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in chicken cone PDE6 GAF-A and GAF-A/B cDNA constructs. *E. coli* XL-blue competent cells were used for transformations, and the mutant cDNAs were purified by QIAprep® spin miniprep kit (Qiagen, Valencia, CA). All mutant cDNAs were sequenced to ensure the proper in-frame subcloning and the desired mutation.

RESULTS

Modeling of the Chicken Cone PDE GAF-A Domain—Sequence alignments between the GAF-A and GAF-B domains of chicken PDE6C to GAF-B of mouse PDE2A showed that the GAF-A domain of PDE6 had very high homology to PDE2A GAF-B and therefore that the basic architecture of the cGMP-binding pocket might be conserved (Fig. 1*A*). Of eight side chains known to contact cGMP in PDE2 GAF-B, five are identical in PDE6C GAF-A, and three of these are in the helix α-4 (H4) of PDE2A GAF-B. In addition, Asp-439 of mouse PDE2A GAF-B is conserved as Glu-124 in chicken PDE6C GAF-A. A three-dimensional homology model of chicken cone PDE6 GAF-A based upon the crystal structure of PDE2A GAF-B domain is shown in Fig. 1*B*. Because of gaps generated by the homology alignment of the two GAF sequences, two extra insertions are introduced as solvent-exposed loops in the modeled structure of chicken cone PDE6 GAF-A. Since both sequences are relatively hydrophobic this does not seem unreasonable. The first insertion resides between the first α-helix and the first β-sheet strand. The second insertion is located between the first and second β-strand. Neither of the two insertions interrupts the secondary structure of the model. For the rest of the sequence, the overall folds of the chicken PDE6C GAF-A domain are very similar to that of mouse PDE2A GAF-B. Finally a putative hydrogen bonding pattern based on the homology model is shown in Fig. 1*C*. This is discussed in more detail later.

The GAF-A Domain Contains the High Affinity cGMP-binding Site—The crystal structure of the mouse PDE2A GAF domains showed that GAF-B but not GAF-A binds cGMP (25). However, the sequence alignment and homology model suggested that in PDE6C, GAF-A should bind cGMP. To test this prediction, three different chicken cone GAF domains, GAF-A, GAF-B, and GAF-A/B, were successfully expressed as soluble proteins in *E. coli*. Each could be purified by Talon resin and gel filtration HPLC to achieve >98% purity (Fig. 2, *A* and *B*). These proteins appeared to be properly folded as they were soluble and eluted as single symmetrical peaks on the sizing column. Binding studies for cGMP on the purified GAF domain proteins showed that both GAF-A/B and GAF-A possessed a single high affinity binding site with K_d values of about 10 nM (Fig. 2, *C* and *D*). However, the GAF-B domain did not bind cGMP (data not shown). Thus, the GAF-A domain appears to contain the only high affinity cGMP-binding site of chicken cone photoreceptor PDE as is also the case for PDE5 (16).

Separation and Identification of Chicken Cone and Rod PDE6—Unfortunately the PDE6C GAF domains only from chicken but not other species (bovine and human) could be expressed as a functional protein in our bacterial system. Therefore, to evaluate the relevance of cGMP binding properties of the bacterially expressed GAF domain, it was essential to understand the cGMP binding characteristics of the chicken PDE6C holoenzyme, which had not previously been studied. Consequently we first needed to identify and isolate chicken

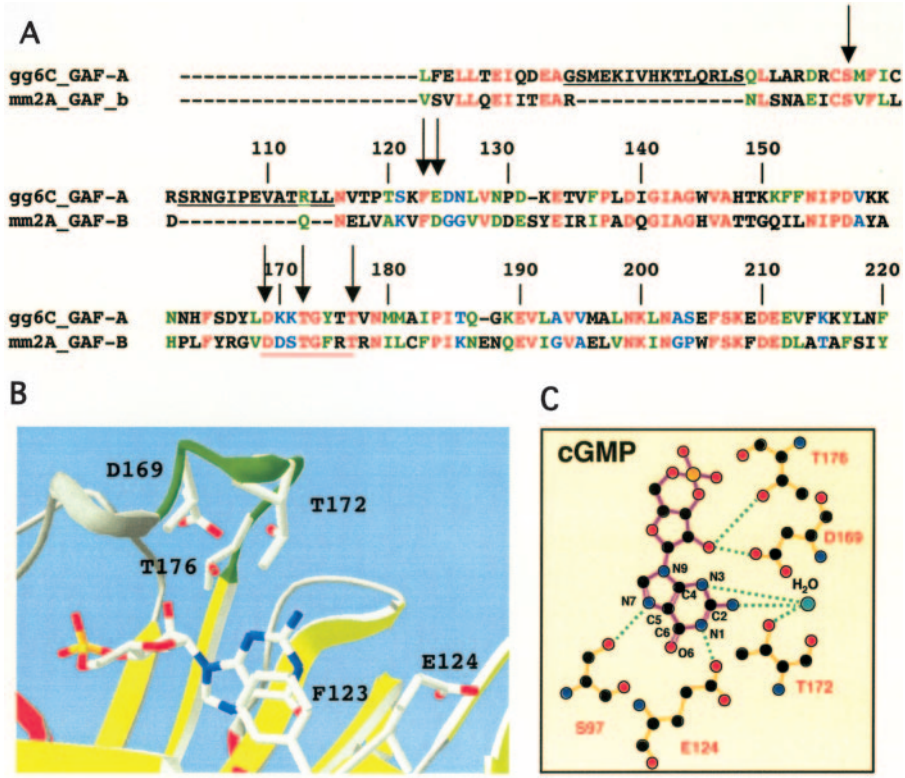


FIG. 1. *A*, sequence homology alignment between chicken cone PDE6 GAF-A domain and mouse PDE2A GAF-B domain. The homology alignment was performed using ClustalW (39). The *black arrows* indicate the 6 conserved residues in close contact with cGMP. Two gaps between the homology sequence are *underlined in black*. The conserved residues of H4 are *underlined in red*. *B*, three-dimensional model of chicken cone PDE6 GAF-A cyclic GMP-binding pocket based on the crystal structure of mouse PDE2A GAF-B. The model was produced using the Swiss-Model program (38). The *green ribbon* indicates the location of H4 of chicken cone PDE6 GAF-A. The model illustrates the position of the residues altered by mutagenesis including the 3 conserved cGMP-contacting residues located on helix 4 (Asp-169, Thr-172, and Thr-176). These residues are oriented toward cGMP and could potentially form hydrogen bonds directly or through a water molecule with cGMP. The phenyl ring of Phe-123 base-stacks with the guanine ring of cGMP. *C*, LigPlot showing probable hydrogen bond interactions with cGMP. gg, *Gallus gallus*; mm, *Mus musculus*; *bt*, *Bos taurus*.

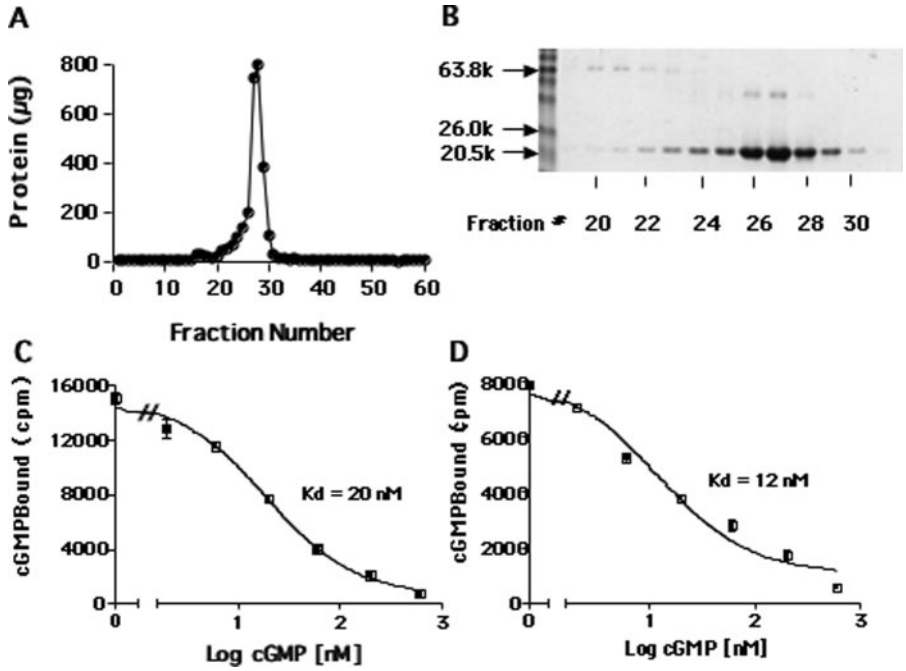


FIG. 2. *A*, superose-12 HPLC purification of chicken cone PDE6 GAF-A. Partially purified chicken cone PDE6 GAF-A domain protein (from the Talon affinity resin) was applied to a Superose-12 HPLC sizing column. The column was run at 0.5 ml/min, and 0.5-ml fractions were collected. The major protein peak eluted at an apparent molecular mass of about 50 kDa corresponding to a GAF-A dimer (assuming a globular shape). *B*, SDS-PAGE of Superose-12-purified chicken cone PDE6 GAF-A. Chicken cone PDE GAF-A domain protein was purified by Talon resin and subsequent Superose-12 sizing column. Superose-12 fractions 20–32 corresponding to the GAF-A protein peak were analyzed by SDS-PAGE and Coomassie Blue staining. The purity of GAF domain proteins was >98% as quantified using NIH Image. *C* and *D*, cGMP binding to chicken cone PDE6 GAF-A and GAF-A/B. cGMP binding competition assays were carried out on chicken cone PDE6 GAF-A and GAF-A/B proteins. 1 nM GAF protein was incubated with 1 nM [^{3}H]cGMP and various amounts of unlabeled cGMP ranging from 2 to 600 nM. The K_d values (determined as IC_{50} values) for cGMP binding to GAF-A and GAF-A/B were 21 ± 4 nM (n = 7) and 10 ± 3 nM (n = 5), respectively. Values are given as the mean ± S.E.

rod and cone PDE6 holoenzyme. Starting with crude extracts from partially dissected chicken retinas, two histone-activated peaks of PDE activity were separated by DE52 anion-exchange chromatography (Fig. 3*A*). Comparison of the chromatography profiles showed that the first and second peaks of activity superimposed with the major peaks of activity from bovine retina (Fig. 3*A*). In the bovine retina these peaks are known to correspond to cone and rod PDE6, respectively (6).

However, the observation that the two PDE6 activities eluted at the same salt gradient concentrations as the bovine

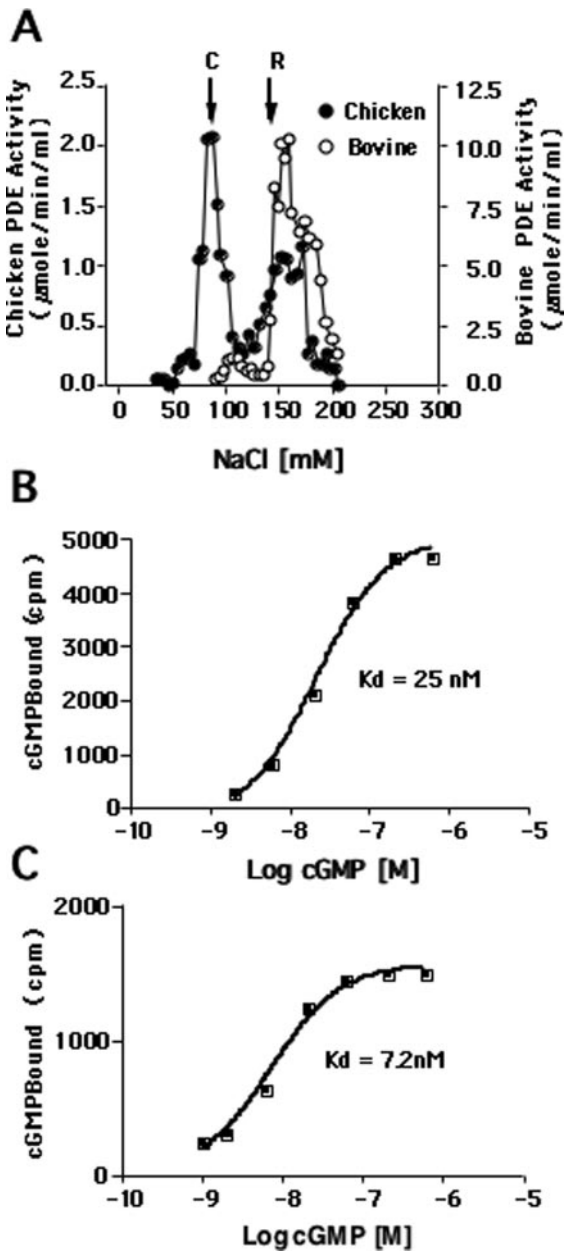


FIG. 3. *A*, DE52 ion-exchange column fractionation of the hypotonic extracts of chicken retina. Methods similar to those described for isolation of bovine PDE6 were used for the chicken PDE6s (44). 100 frozen chicken retinas were extracted with hypotonic buffer (10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). After 100,000 × *g* centrifugation, the retinal supernatant was applied to a 75-ml DE52 ion-exchange column equilibrated in the hypotonic buffer plus 20 mM NaCl and eluted with a linear NaCl gradient (20–300 mM) run at 1 ml/min at 4 °C. A total of 60 fractions of 5 ml each was collected and assayed for PDE activity in the presence of 2.5 mg/ml histone. There are two histone-activated PDE6 activity peaks: rod (*R*) and cone (*C*) PDE6 peaks. *B* and *C*, cGMP binding to chicken cone (*B*) and rod (*C*) PDE6 holoenzyme. Chicken rod or cone PDE6 holoenzyme was immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G PLUS-agarose resin as described under "Methods." The immunoprecipitated enzyme was incubated with cGMP binding buffer in the presence of 2 mM EDTA, 1 mM IBMX, and 2–600 nM (7.5 Ci/mmol) [^{3}H]cGMP in a total volume of 200 μl for 2 h at room temperature. After incubation, samples were subjected to a filter binding assay. The data shown here are from a single representative experiment that was repeated three times. The mean values were 25 ± 5 nM for cone and 7 ± 3 nM for rod PDE6 (mean ± S.E.).

cone and rod PDE6 isozymes did not unambiguously verify the identity of the first peak as the chicken cone and the second peak as the chicken rod enzyme. Therefore, each peak was further analyzed by cGMP affinity column chromatography or by immunoprecipitation followed by mass spectrometry. Only the first peak bound to a cGMP affinity column as is the case for the bovine cone isoenzyme (7). However, both peaks could be absorbed to the ROS-3 antibody. The presence and purity of the chicken photoreceptor PDE6 in either the eluate from the cGMP affinity column or the immunoprecipitated pellets were assessed by silver or Coomassie stain of SDS-polyacrylamide gels (Fig. 4, *A1* and *B1*).

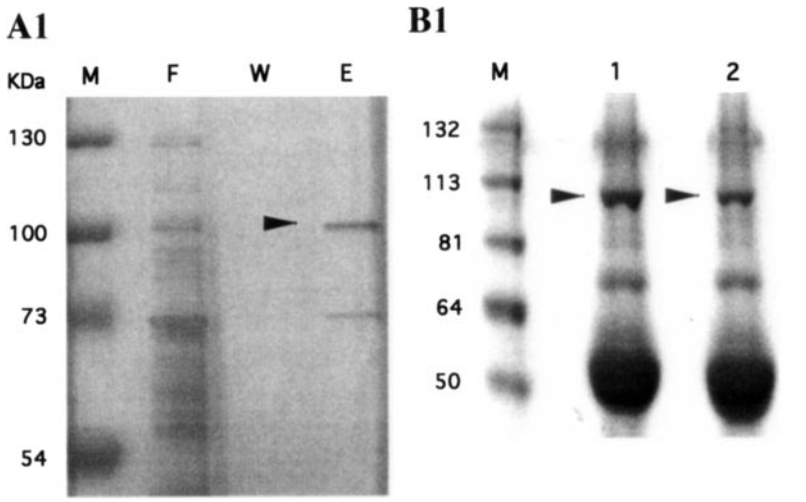
To positively identify the first peak as chicken cone PDE6, the protein eluted from the cGMP affinity column was trypsin-digested and analyzed by mass spectrometry. Thirteen peptides larger than 2 amino acids from the MS/MS spectra were exact matches with the chicken cone PDE6 α′-subunit. No other large PDE peptides were identified. Thus, the first histone-activated PDE6 activity peak off the DE52 ion-exchange column contains chicken cone PDE6 (Fig. 4*A2*).

However, since the putative rod PDE6 did not bind to the cGMP affinity column and could not be purified by this method, both PDE activity peaks from the DE52 column were further purified by immunoprecipitation with ROS-3 monoclonal antibody. In this case, the immunoprecipitation pellets were separated by SDS-PAGE and stained with Coomassie Blue. The protein bands corresponding in size to the major subunits of PDE6 were trypsin-digested and analyzed by mass spectrometry (Fig. 4*B1*).

Seventy MS/MS spectra were acquired during a 45-min liquid chromatography/MS/MS run using data-dependent selection of the doubly, triply, and quadruply charged peptide precursors. Upon manual inspection 37 low quality MS/MS spectra with ion intensities below 20 counts were discarded. The remaining 33 MS/MS spectra were searched against the nonredundant National Center for Biotechnology Information protein data base using the MASCOT search engine. The search produced 12 matches to tryptic fragments of chicken rod PDE6B and nine matches to common protein contaminants (keratins and trypsin). The remaining 12 good quality MS/MS spectra (with ion intensities of at least 20 counts) were subjected to manual *de novo* sequencing, and all of them could be assigned to chicken rod PDE6B peptides. These peptides were not identified by the initial data base search because four contained oxidized methionines, two were non-tryptic fragments, and the other three had more than one missed cleavage. In addition, one peptide contained an acrylamide-modified cysteine, and the N-terminal peptide was shown to be acetylated by the corresponding MS/MS spectra recorded for the doubly and triply charges ions of this peptide. Based on these data we conclude that chicken rod PDE6B was the only major protein contained in the gel band. The protein coverage obtained by MS/MS is shown in Fig. 4*B2*.

In addition, the MASCOT search result indicated that 16 peptides from the putative cone PDE6 protein band (peak 1) were exact matches with chicken cone α′-subunit (PDE6C) (data not shown). Therefore, we confirmed our earlier observation that the first histone-activated PDE6 activity peak contained the chicken cone PDE6C.

The firm identification of the chicken cone and rod enzymes allowed us to compare the cGMP binding characteristics of the holoenzymes in the DE52 fractions with those of the purified individual GAF domains described earlier. For this we used an immunoprecipitation method for measurement of cGMP binding to rod and cone PDEs (6, 30). This method was used to ensure that all binding measured was due only to the PDE6 and not other nucleotide-binding proteins likely to be in the DE52 fractions. Pilot experiments with purified chicken GAF domains (not shown) and earlier studies with bovine PDE6



A2

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MGEVNKDAVEK-YLENNPQFAK-EYFDRK-MR-AEVLGSIFQVSPGDVK-EGVSF 50
K-DMSR-LEECNILFELLTEIQDEAGSMEK-IVHK-TLQR-LSQLLAR-DR-CSM 98
FICR-SR-NGIPEVATR-LLNVTPTSK-FEDNLVNPDK-ETVFPLDIGIAGWVAH 148
TK-K-FFNIPDVK-K-NNHFSDYLDK-K-TGYTTVNMMAIPITQGK-EVLAVVMA 196
LNK-LNASEFSK-EDEEVFK-K-YLNFISLVLR-NHHTSYLYNIESR-R-SQMLL 244
WSANK-VFEELTDIER-QFHK-ALYTIR-MYLNCER-YSVGLLDMTK-EK-EFYD 292
EWPIR-LGEAEPYK-GPK-TPDGR-EVNFYK-IIDYILHGK-EEIK-VIPTPPAD 340
HWCLISGLPTYVAENGFICNMMNAPADEYFTFQK-GPVDETGWVIK-NVLSLPIV 393
NK-K-EEIVGVATFYNR-K-DGK-PFDEYDEQIIETLTQFLGWSVLNTDTYDK-M 442
NK-LENR-K-DIAQEMLMYQTK-ATPTEVESILK-YK-EK-LNVK-SIEECDEK- 488
DLIRILK-EELPDPK-DLELYEFR-FSDFPVTEHGLITCGIR-LFFEINVVEK-F 538
K-VPAEVLTR-WMYTVR-K-GYR-DITYHNWR-HGFNVGQTMFTLLMTGR-IK-K 585
-YYTDLEAFAMVAAAFCHDIDHR-GTNNLYQMK-SAAPLAK-LHGSSILERHHLE 636
YSK-TLLQDESLNIFQNLNK-RQFETVLHLFEVAIIATDLALYFK-K-R-TMFQK 686
-IVDAIEK-METEEEAIK-YISIDPTK-K-EVIMAMMMTGCDLSAITK-PWEVQS 735
K-VALMVANEFWEQGDLER-TVLQQQPIPMMDR-NK-GDELPK-LQVGFIDFVCT 785
FVYK-EFSRFHK-EITPMFDGLQNNR-VEWK-TR-ADEYEEK-MK-VIEEQK-K- 831
K-EEEAAAK-K-AENAAGGGGGGEDGK-SK-TCIVL
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B2

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MSISEDDVEK-FLDGNPAFAK-QYFEK-K-LK-TESCDDNESEILFELIQDMQES 50
INMEK-VVFK-TLR-R-IR-SLIHADR-CSLFMYR-QR-NGTPELATR-LFNIQE 96
GSTLEECLVSPDCEIVYPLDLGIVGYVAQTK-K-TMNIK-DVSECPQFSSFVDEL 148
TDYTTK-SILATPILNGK-DLVAVILAINK-LNGPFFTNSDETLFLK-YLNFASL 199
NLK-IYHLSYLHNCETR-R-GQVLLWSANK-VFEELTDIER-QFHK-AFYTVR-A 247
YLNCDR-YSVGLLDMTK-QK-EFFDLWPVLLGEVPPYSGPR-TPDGR-EIVFYK- 296
VIDYILHGK-EDIK-VIPNPTPDHWALVTGLPTYVAESGFICNIMNAAADEMFNF 349
QEGPLDESGWTIK-NVLSMPIVNK-K-EEIVGVVTFFNR-K-DGK-PFDEQDETL 398
MESLTQFLGWSVLNTDTYDK-MNK-LENR-K-DIAQDMVLYHVK-CDK-DEIQEI 447
LPTR-EK-LGK-EPSECEEEELASILK-EQLPGPTK-FEIYEFR-FSDFDCTELE 496
LVK-CGIQMYYELGVVK-K-FQIPQEVLVR-FVYSVSK-GYR-K-ITYHNWR-HG 543
FNVAQTMFTLLMTGK-LK-R-YYTDLEAFAMVTAALCHDIDHR-GTNNLYQMK-S 593
QNPLAK-LHGSSILER-HHLDFGK-FLLSEESLNICQNLNR-R-QHEHMIHLMDI 643
AIIATDLALYFK-K-R-TMFQK-IVDESK-TYDNTTAWTDYLSLETTK-K-EVVM 691
AMMMTACDLSAITK-PWEVQSK-VALLVAAEFWEQGDLEISVLQQQP
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FIG. 4. *A1*, SDS-PAGE analysis of chicken cone PDE6 purified by cGMP affinity chromatography. Seven fractions from the first peak of the DE52 ion-exchange chromatography (35 ml) were pooled together and loaded on an epoxy-Sepharose cGMP affinity column (15). After washing with 15 ml (5× bed volume) wash buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 2 mM EDTA, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM IBMX). The cGMP-binding proteins including chicken cone PDE6 were eluted with 1 mM cGMP at the presence of 2 mM EDTA and 1 mM IBMX. The purity of the eluted protein fractions was analyzed by silver staining the SDS-polyacrylamide gels (*M*, marker; *E*, eluate; *W*, wash; *F*, flow-through). The *arrowhead* indicates the protein band of chicken cone PDE6 α'-subunit. *A2*, MS/MS coverage of chicken cone PDE6 α'-subunit. Of 862 amino acids from the protein sequence (GenBank™ accession number I50186), 156 amino acids (shown in *bold type*) were verified by MS/MS, indicating an 18% protein coverage. "_" indicates the probable trypsin cleavage site. *B1*, SDS-PAGE analysis of immunoprecipitated chicken rod and cone PDE6 holoenzyme. Chicken rod or cone PDE6 holoenzymes were immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G-Sepharose resin. The pellets were analyzed by Coomassie staining of the SDS-polyacrylamide gel (*M*, marker; *lane 1*, first PDE6 activity peak off DE52 column; *lane 2*, second PDE6 activity peak off DE52 column. The *arrowheads* indicate the protein bands of chicken cone and rod PDE6. *B2*, MS/MS coverage of chicken rod PDE6 β-subunit. Of 736 amino acids from the protein sequence, 249 amino acids (shown in *bold type*) were verified by MS/MS, indicating a 34% protein coverage. "_" indicates the probable trypsin cleavage site. Note several sequences for chicken PDE6B that do not entirely agree with each other have been deposited in GenBank™. The sequence shown here (GenBank™ accession number AAO67732) represents the sequence with the highest number of exact matches with the MS/MS data.

holoenzymes indicate that this antibody does not alter the binding properties for cGMP compared with the Millipore method (30). [^{3}H]cGMP was found to bind to a single class of high affinity sites on chicken cone PDE6 with a K_d of 25 ± 5 nM (Fig. 3*B*). The apparent cGMP binding affinity to the rod PDE6 was slightly higher than that of cone with an apparent K_d value of 7 ± 3 nM (Fig. 3*C*). To prevent hydrolysis of cGMP by the holoenzyme, the immunoprecipitated PDE was incubated with [^{3}H]cGMP in the presence of 1 mM IBMX and 2 mM EDTA.

The bovine cone PDE6C holoenzyme is reported to exhibit a single class of high affinity cGMP-binding sites with a K_d of about 11 nM (6). Thus, chicken cone PDE6C has very similar non-catalytic cGMP binding characteristics to bovine cone PDE6C. In addition, the bacterially expressed chicken cone PDE6C GAF-A/B and GAF-A domains each bound cGMP with K_d values of ~10–20 nM. This is in good accordance with the K_d value of the holoenzyme suggesting that the isolated GAF domain proteins provide a reasonable model system for studying the cGMP binding properties of the native enzyme.

Enzyme Kinetics of Chicken Rod and Cone PDE6—Using the DE52 rod and cone fractions, the kinetic values for both cGMP and cAMP hydrolysis by chicken rod and cone PDE6 were determined (Table I). The K_m values were 29 ± 9 and 26 ± 5 μM for cGMP and 820 ± 34 and 717 ± 44 μM for cAMP for the rod

TABLE I
Apparent K_m values for chicken and bovine PDE6 holoenzyme

The K_m values for both cGMP and cAMP hydrolysis by chicken rod and cone PDE6 were determined. The data for bovine rod and cone PDE6s were published previously in our laboratory (6). Data are presented as the means ± S.D.

	Chicken		Bovine[a]	
	Cone	Rod	Cone	Rod
$K_{m(cGMP)}$ (μM)	26 ± 5 (n = 3)	29 ± 9 (n = 3)	17 ± 3 (n = 6)	17 ± 7 (n = 5)
$K_{m(cAMP)}$ (μM)	717 ± 44 (n = 3)	820 ± 34 (n = 3)	610 ± 50 (n = 4)	ND[b]

[a] Data from Gillespie *et al.* (6).
[b] Not determined.

and cone enzymes, respectively. These values are very similar to those determined previously for bovine PDE6s (Table I).

Residues Critical for cGMP Binding Affinity and Specificity—As noted earlier, there are 6 residues that directly bind cGMP in PDE2 that are identical or highly conserved in PDE6C. To determine the relative contribution of each residue to binding, each of the 6 conserved amino acids was mutated to alanine. Each of these mutations had a negative effect on the cGMP binding affinity (Fig. 5 and Table II). Both the S97A and E124A mutants decreased cGMP binding affinity about 5-fold (Fig. 5). The other four alanine mutants lost cGMP binding capability completely as shown in Table II.

In mouse PDE2A GAF-B, the side chain of Phe-438 is base-stacked with the guanine ring of cGMP through π-π interactions. Phe-123 of chicken PDE6C corresponds to Phe-438 of mouse PDE2A, and the homology model suggested that the phenyl ring of Phe-123 might form a similar interaction in PDE6C (Fig. 1*B*). When Phe-123 was mutated to either alanine or tryptophan, cGMP binding was undetectable up to 100 μM [^{3}H]cGMP. However, when Phe-123 was mutated to tyrosine, the K_d for cGMP binding was about 40 nM, very similar to wild-type protein. Thus, it appears likely that in cone PDE6C a π-π interaction between the phenyl ring of Phe-123 and the guanine ring of cGMP is formed and is essential for stabilizing cGMP binding to the chicken cone PDE6C GAF-A domain.

H4 and cGMP Binding—To date, two GAF domain structures have been elucidated by x-ray crystallography, mouse PDE2A (25) and yeast YKG9 protein (41). The GAF domain of YKG9 has no cGMP binding capability. Nevertheless the overall folds of these two GAF domains are very similar. However, in PDE2A GAF-B, between β-strands 4 and 5, there is an additional helix (H4) containing 3 amino acid residues that make side chain contact with bound cGMP (Fig. 1*A*; Asp-169, Thr-172, and Thr-176). All 3 residues are conserved in the GAF-A domains of PDE6A, PDE6B, and PDE6C and also in PDE5A. When any of these residues were mutated to alanine, the GAF domain completely lost its ability to bind cGMP (Table II and Fig. 1*C*). As a control, alanine mutations also were introduced into the solvent-exposed residues (Ser-165, Asp-166, Lys-170, and Thr-175) of H4 of the homology model. Each of these alanine mutants retained most of the binding activity of wild-type GAF protein (Table II) although these residues are in close proximity to the cGMP-interacting amino acids. Based on the above observations, we propose that H4 is essential for cGMP binding and that interactions between each of the 3 conserved amino acid side chains with cGMP is necessary for high affinity nucleotide binding.

Cyclic Nucleotide Selectivity of Chicken Cone PDE6 GAF Domain—In contrast to its high affinity binding to cGMP, the chicken cone PDE6 GAF domain has extremely low affinity for cAMP with an apparent affinity of 35 ± 11 mM (Fig. 6). The crystal structure of mouse PDE2A GAF-A/B suggests that Asp-439 provides a positive specificity determinant for cGMP binding due to hydrogen bonds between its side chain carboxylate with the O-6 and N-1 of the guanine base of cGMP (25, 28). This residue also has a negative effect on cAMP binding in PDE2A

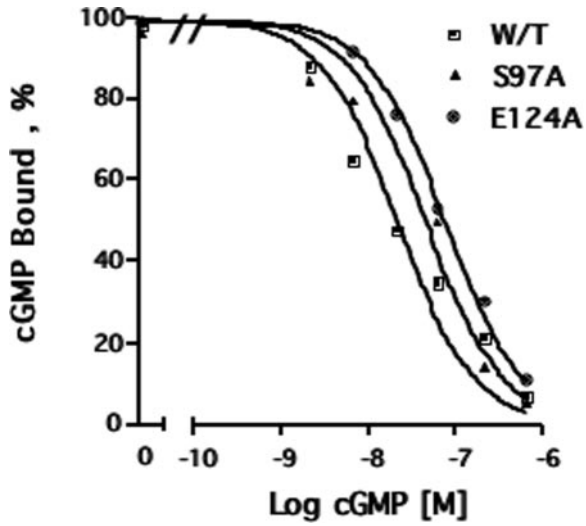

FIG. 5. **cGMP binding to chicken cone PDE6 GAF-A/B wild type, S97A mutant, and E124A mutant.** cGMP binding competition assays were carried out as in Fig. 4. Wild type (*closed squares*) and two mutants, S97A (*closed triangles*) and E124A (*closed circles*) are shown. Figure was plotted using Prism4 constraining the 100 and 0% binding points. A representative experiment is shown. The experiment was repeated three times, and the mean values ± S.E. were 57 ± 9 nM for E124A, 63 ± 7 nM for S97A, and 10 ± 3 nM for wild type (*W*/*T*).

(28). Residue Glu-124 of chicken PDE6C GAF-A corresponds to Asp-439 of mouse PDE2A GAF-B. Thus, Glu-124 of chicken PDE6C GAF-A was predicted to contribute to the cGMP binding specificity of PDE6C. Indeed the alanine mutant E124A showed a 5-fold decrease in cGMP affinity and 30-fold increase in cAMP affinity (Fig. 6). However, it is clear that other additional specificity determinants also must be present as the mutant protein still maintained a greater than 1000-fold selectivity for cGMP.

DISCUSSION

Identification of Chicken Rod and Cone PDEs—In contrast to most species, chickens are known to have cone-dominated retinas with up to 85% of the photoreceptors as cones (42). The DE52 elution profile, histone activation, and kinetic properties of the chicken photoreceptor PDEs show that the holoenzymes have enzyme characteristics very similar to the comparable photoreceptor PDEs of other species such as bovine, human, and frog. In addition, direct binding studies on the isolated chicken PDE6C cone GAF domains show that they have very similar non-catalytic cGMP binding properties of the holoenzyme. These results suggest that the basic enzymatic and cGMP binding characteristic have been greatly conserved between chicken and bovine PDE6s during evolution and that the regulation and properties of the chicken PDE6C GAF domains reported in this study are likely to be relevant to those of mammalian species.

Subunit Structure of Chicken Rod PDE6—Rod photoreceptor PDE6s of various species are thought to be composed of a heterodimer containing two distinct major catalytic subunits:

TABLE II

Effect on cGMP binding affinity by mutation of residues lining the cGMP-binding pocket

Column 2, apparent K_d values for each construct, including wild type and mutants, were derived from at least three individual cGMP binding experiments with duplicate points and expressed as mean ± S.D. Column 3, atom in residue of column 1 closest to the cGMP. CZ, carbon-ζ ; OD, oxygen-δ ; OG, oxygen-γ . Column 4, the distance between cGMP and the amino acid side chain of mouse PDE2A GAF-B domain. For wild type (W/T) GAF-A/B, $n = 5$; for the mutants, $n = 3$. aa, amino acid; gg, *G. gallus*; NB, no binding; H4, located on H4 of GAF-A.

aa residue in ggPDE6C GAF-A/B	K_d for cGMP	Atom of W/T residue in closest contact with cGMP	Distance of interacting atom from cGMP
	nM		*Å*
W/T	10 ± 3		
Mutations in residues interacting with guanine ring			
F123A	NB	CZ	3.2
F123W	NB	CZ	3.2
F123Y	38 ± 5	CZ	3.2
E124A	57 ± 9	OD1 or OD2	2.7
T172A (H4)	NB	OG1	3.2
Mutations in residues interacting with phosphate-ribose or imidazole ring			
S97A	63 ± 7	OG	2.7
D169A (H4)	NB	OD2	2.9
T176A (H4)	NB	OG1	2.8
Mutations in residues not interacting directly with cGMP			
S165A (H4)	103 ± 11		
D166A (H4)	178 ± 27		
K170A (H4)	70 ± 12		
T175A (H4)	79 ± 9		

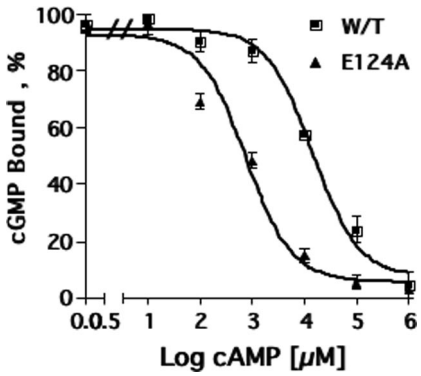

FIG. 6. **cAMP binding of GAF-A wild type and E124A mutant.** cAMP binding competition assays were carried out on chicken cone PDE6 GAF-A wild-type (*W/T*) and E124A mutant proteins. 1 nM GAF protein was incubated with 1 nM [^{3}H]cGMP and various amounts of unlabeled cAMP ranging from 1 μM to 100 mM. The IC_{50} values (mean ± S.D.) for cAMP binding were 35 ± 11 mM ($n = 3$) and 1.0 ± 0.3 mM ($n = 4$) for the wild-type GAF-A and E124A mutants, respectively. Figure was plotted as described in Fig. 5.

the α- and β-subunits. However, our mass spectrometry analysis of the enzyme of the second DE52 peak from chicken retina only detected the β-subunit of rod PDE6 (PDE6B). Initially we thought that this was due to the fact that only the α′-subunit of chicken cone (PDE6C) and β-subunit of chicken rod (PDE6B) were in the data base (10, 11). However, when all of the acquired MS/MS spectra were searched against all the available PDE6A sequences from all species in the National Center for Biotechnology Information data base, no unique PDE6A peptide matches were found. Therefore, one must consider the possibility that the rod enzyme in chicken does not contain an α-subunit, and its absence may be a unique characteristic of chicken rod photoreceptor PDE6. It is probably worth noting that among all the expressed sequence tag cDNAs in the chicken data base, there are none that are most homologous to α-subunit of any other species. Both observations taken together strongly suggest that the chicken rod holoenzyme is a homodimer composed of two β-subunits. The functional significance of this difference in structure remains to be determined.

Specificity for Cyclic Nucleotide Binding to the GAF-A Domain of Photoreceptor PDEs—cGMP and cAMP are composed of phosphate-ribose and purine base moieties. cGMP and cAMP differ at their purine rings and have different hydrogen bonding potential in the C-2 and C-6 positions of these rings. By using cGMP analogs, Cote's group (29) has suggested that interaction between GAF domain residues and the N-1/O-6 region of the purine ring of cGMP determines the specificity of cGMP binding of frog PDE6. This proposition was reinforced by the crystal structure of the mouse PDE2 GAF domain. Indeed Asp-439 of mouse PDE2A is located in the GAF-B cGMP-binding pocket and forms two hydrogen bonds; one is between the main chain NH and O-6 of the guanine ring, and the other is between the side chain carboxylate and the N-1 position of cGMP (25).

The affinities for cGMP determined for the individual bacterially expressed chicken PDE6C GAF domains are very similar to those reported previously for PDE2A and PDE5A with K_d values around 10 nM (28). Since mutation of the corresponding conserved residues in PDE6C reduces cGMP affinity, these data strongly support the validity of the homology model of PDE6C GAF-A shown in Fig. 1*B*. Furthermore they suggests a high similarity of the overall binding pocket structure between PDE2A, PDE5A, and PDE6C GAF domains. However, this homology model does not explain the differences in cyclic nucleotide selectivity between PDE6C and PDE2A or PDE5A. In PDE2A, 3 residues including Phe-438, Asp-439, and Thr-488 have been demonstrated to each be necessary for cAMP/cGMP selectivity (28). Only 1 residue corresponding to Glu-124 in PDE6C has been shown in the present study to participate in nucleotide discrimination. More importantly, the modeled structure of PDE6C GAF-A does not elucidate what additional molecular determinants might be likely to produce the much greater cyclic GMP selectivity of PDE6C compared with PDE2A and PDE5A. It has been shown previously that cAMP has very little binding affinity to the non-catalytic cGMP-binding site of frog PDE6 (29). The same observation was obtained with our bacterially expressed chicken cone PDE6 GAF proteins. We find nearly a 100,000-fold decreased affinity for cAMP compared with cGMP in the isolated GAF-A constructs. The PDE2A and PDE5A GAF domains are reported to have about 20- and 1000-fold greater selectivity, respectively, for

cGMP *versus* cAMP, while the chicken PDE6C holoenzyme has more than 100,000-fold greater selectivity (29, 43). It is also known that a D439A mutation of PDE2A significantly increases its cAMP binding affinity resulting in similar affinities for both cAMP and cGMP (28). In the present study the analogous E124A mutant in PDE6 caused an ~30-fold increased affinity for cAMP compared with wild type (Fig. 6). Nevertheless its absolute binding affinity for cAMP is still more than 1000-fold lower than that for cGMP. Therefore, there must be additional structural components conferring negative selectivity for cAMP only for the photoreceptor PDEs. It will likely require the direct determination of the structure of the PDE6C GAF-A to learn the molecular basis for this selectivity in the photoreceptor PDEs.

To our knowledge there have been no previous reports of high yield bacterial expression of functional bovine PDE6 either in its entirety or of its individual domains, although good expression of several PDE5/PDE6 chimeric molecules has been reported (31). Therefore, the robust bacterial expression of chicken cone PDE6 GAF domains has substantial importance in that it has allowed these basic characterization studies to be carried out. Moreover, since the characterization of the chicken PDE6 holoenzymes suggested that the kinetics and binding properties are greatly conserved between chicken, bovine, and frog isozymes, our understanding of the regulatory properties learned from analysis of these domains is likely to be widely applicable. It is hoped that they will facilitate new insights into the function and evolution of this unique domain as an essential unit of various signaling and sensory transducers.

REFERENCES

1. Palczewski, K., and Saari, J. C. (1997) *Curr. Opin. Neurobiol.* **7,** 500–504
2. Helmreich, E. J., and Hofmann, K. P. (1996) *Biochim. Biophys. Acta* **1286,** 285–322
3. Pugh, E. N., and Cobbs, W. H. (1986) *Vision Res.* **26,** 1613–1643
4. Baylor, D. A. (1987) *Investig. Ophthalmol. Vis. Sci.* **28,** 34–49
5. Deterre, P., Pfister, C., Bigay, J., and Chabre, M. (1987) *Biochimie* (*Paris*) **69,** 365–370
6. Gillespie, P. G., and Beavo, J. A. (1988) *J. Biol. Chem.* **263,** 8133–8141
7. Gillespie, P. G., and Beavo, J. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4311–4315
8. Florio, S. K., Prusti, R. K., and Beavo, J. A. (1996) *J. Biol. Chem.* **271,** 24036–24047
9. Morris, V. B., and Shorey, C. D. (1967) *J. Comp. Neurol.* **129,** 313–340
10. Semple-Rowland, S. L., and Green, D. A. (1994) *Exp. Eye Res.* **59,** 365–372
11. Morin, F., Lugnier, C., Kameni, J., and Voisin, P. (2001) *J. Neurochem.* **78,** 88–99
12. Beavo, J. A. (1995) *Physiol. Rev.* **75,** 725–748
13. Soderling, S. H., and Beavo, J. A. (2000) *Curr. Opin. Cell. Biol.* **12,** 174–179
14. Aravind, L., and Ponting, C. P. (1997) *Trends Biochem. Sci.* **22,** 458–459
15. Martins, T. J., Mumby, M. C., and Beavo, J. A. (1982) *J. Biol. Chem.* **257,** 1973–1979
16. Francis, S. H., Bessay, E. P., Kotera, J., Grimes, K. A., Liu, L., Thompson, W. J., and Corbin, J. D. (2002) *J. Biol. Chem.* **277,** 47581–47587
17. Rybalkin, S. D., Rybalkina, I. G., Shimizu-Albergine, M., Tang, X. B., and Beavo, J. A. (2003) *EMBO J.* **22,** 469–478
18. Corbin, J. D., Blount, M. A., Weeks, J. L., II, Beasley, A., Kuhn, K. P., Ho, Y. S., Saidi, L. F., Hurley, J. H., Kotera, J., and Francis, S. H. (2003) *Mol. Pharmacol.* **63,** 1364–1372
19. Zoraghi, R., Corbin, J. D., and Francis, S. H. (2004) *Mol. Pharmacol.* **65,** 267–278
20. Cote, R. H., Bownds, M. D., and Arshavsky, V. Y. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4845–4849
21. Yamazaki, M., Li, N., Bondarenko, V. A., Yamazaki, R. K., Baehr, W., and Yamazaki, A. (2002) *J. Biol. Chem.* **277,** 40675–40686
22. D'Amours, M. R., and Cote, R. H. (1999) *Biochem. J.* **340,** 863–869
23. Mou, H., Grazio, H. J., III, Cook, T. A., Beavo, J. A., and Cote, R. H. (1999) *J. Biol. Chem.* **274,** 18813–18820
24. Mou, H., and Cote, R. H. (2001) *J. Biol. Chem.* **276,** 27527–27534
25. Martinez, S., Wu, A., Glavas, N., Tang, X., Turley, S., Hol, W., and Beavo, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13260–13265
26. Liu, L., Underwood, T., Li, H., Pamukcu, R., and Thompson, W. J. (2002) *Cell. Signal.* **14,** 45–51
27. Sopory, S., Balaji, S., Srinivasan, N., and Visweswariah, S. S. (2003) *FEBS Lett.* **539,** 161–166
28. Wu, A. Y., Tang, X. B., Martinez, S. E., Ikeda, K., and Beavo, J. A. (2004) *J. Biol. Chem.* **279,** 37928–37938
29. Hebert, M. C., Schwede, F., Jastorff, B., and Cote, R. H. (1998) *J. Biol. Chem.* **273,** 5557–5565
30. Gillespie, P. G. (1988) *Identification, Purification, and Characterization of Bovine Rod and Cone Photoreceptor Phosphodiesterase Isozymes*. Ph.D. thesis, University of Washington
31. Granovsky, A. E., Natochin, M., McEntaffer, R. L., Haik, T. L., Francis, S. H., Corbin, J. D., and Artemyev, N. O. (1998) *J. Biol. Chem.* **273,** 24485–24490
32. Hurwitz, R. L., Bunt-Milam, A. H., and Beavo, J. A. (1984) *J. Biol. Chem.* **259,** 8612–8618
33. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68,** 850–858
34. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* **20,** 3551–3567
35. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108
36. Rovati, G. E. (1998) *Trends Pharmacol. Sci.* **19,** 365–369
37. Yamazaki, A., Sen, I., and Bitensky, M. W. (1980) *J. Biol. Chem.* **255,** 11619–11624
38. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18,** 2714–2723
39. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
40. Way, M., Pope, B., Gooch, J., Hawkins, M., and Weeds, A. G. (1990) *EMBO J.* **9,** 4103–4109
41. Ho, Y. S., Burden, L. M., and Hurley, J. H. (2000) *EMBO J.* **19,** 5288–5299
42. Morris, V. B. (1970) *J. Comp. Neurol.* **140,** 359–398
43. Francis, S. H., Thomas, M. K., and Corbin, J. D. (1990) in *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action* (Beavo, J., and Houslay, M. D., eds) Vol. 2, pp. 117–140, John Wiley & Sons, Chichester, UK
44. Cook, T. A., and Beavo, J. A. (2000) *Methods Enzymol.* **315,** 597–616